STUDIES ON OXIDATION AND REDUCTION BY PNEUMOCOCCUS.

III. Reduction of Methylene Blue by Sterile Extracts of Pneumococcus.

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The two preceding papers of this series have dealt with the phenomenon of peroxide formation by pneumococcus. The first of these studies (1) concerned itself with the conditions under which this compound is formed when air is admitted to cultures of pneumococci which have been grown anaerobically. Under these conditions peroxide is formed at reactions and at temperatures inhibiting active cell multiplication. These facts suggest that the mechanism of peroxide production is dependent upon an interaction of oxygen and cell constituents, and that under experimental conditions, at least, this reaction may proceed independently of cell growth. This view is further supported by the data presented in the second paper (2) on the formation of peroxide by sterile extracts of pneumococci. The fact that soluble extracts of pneumococcus cells form peroxide when exposed to molecular oxygen is convincing evidence that this function is not dependent upon the presence of living, formed cells.

Just as the admission of air to sterile pneumococcus extracts was found to give rise to the prompt formation of peroxide, so in the present experiments it will be shown that the addition of methylene blue to these same extracts results in rapid reduction of the dye to its colorless base. The admission of air or the addition of dye to pneumococcus extracts, in the absence of living cells, induces reactions which although yielding dissimilar products, may be alike in nature, depending upon whether molecular oxygen or methylene blue serves as hydrogen acceptor.

In the following experiments, certain conditions are defined which influence the reduction of methylene blue by pneumococcus extracts; and certain similarities are pointed out which serve to relate dye reduction and peroxide formation as functions of the same or closely allied oxidation-reduction systems in the cell.

EXPERIMENTAL.

Methods.

Preparation of Extracts.—The sterile extracts of pneumococci used in the present experiments were prepared according to the methods previously described (2). They consisted either of broth extracts of unwashed pneumococci or of washed cells extracted in phosphate solution.

Technique of Methylene Blue Reduction Tests.—A stock solution of Merck's medicinal methylene blue, in concentration of 1:1,000 in water, was sterilized in the autoclave and stored in the ice box. At the time of testing, dilutions of the stock solution were freshly prepared in sterile 0.1 M phosphate solution, pH 7.5. Exhaustion of air from the test fluids was not carried out prior to the addition of the extract, but the amount of molecular oxygen present was kept uniform throughout by saturating all solutions with air at a constant temperature of 25°C., and further admission of air was prevented by sealing the tubes with vaseline. The sterile extracts of pneumococci used in the tests were treated as indicated in the protocols. The solutions containing the complete reduction system were placed in narrow tubes of uniform bore, sealed with vaseline, and incubated at 37°C.

Reduction of Methylene Blue by Sterile Broth Extracts of Unwashed Pneumococci.[1]

In repeated experiments it has been found that small amounts of the sterile cell extract possess the property of rapidly reducing large amounts of methylene blue. For example, 0.3 cc. of the sterile extract added to 1 cc. of 1:20,000 methylene blue solution effected complete reduction in 40 minutes at 37°C.; the same amount of sterile extract added to 1 cc. of 1:2,000 methylene blue (a concentration of approximately 1 mM) brings about complete reduction in $2\frac{1}{2}$ hours.

Peroxide may be considered as a product resulting from the transfer of hydrogen to molecular oxygen in the same way that the leuco

[1] Unless otherwise specified, the term pneumococcus extract refers to broth extracts of unwashed cells.

base of methylene blue may be regarded as a product resulting from the transfer of hydrogen to the dye. Thus, from the nature of the processes involved in peroxide formation and methylene blue reduction, it is possible that the same system or systems in the extract may be responsible for both activities; if molecular oxygen is admitted to an active extract peroxide is formed, if methylene blue is added to the same extract, methylene white, or the leuco base of the dye, is formed. In both cases, some constituent of the extract is oxidized, with the simultaneous formation of peroxide or of methylene white, depending merely upon whether molecular oxygen or methylene blue serves as hydrogen acceptor.

With these relationships in mind, the effects of various agents on the activity of these two functions of pneumococcus extracts have been investigated in the following experiments.

Effect of Heat upon Methylene Blue Reduction by Extracts of Pneumococcus.

In order to determine the effect of heat upon the reducing action of pneumococcus extracts, the following experiments were carried out. For purposes of comparison the heat sensitiveness of the peroxide-forming and methylene blue-reducing activities of the same extract was studied under similar conditions.

1. Influence upon the Potency of the Extracts of 5 Minutes Exposure to Temperatures Varying from 55–100°C.—In the following experiment a broth extract of unwashed pneumococci was heated for 5 minutes at the stated temperatures. The ability of the heated extract to reduce methylene blue and form peroxide was then determined.

1.5 cc. portions of cell extract were placed in a series of small, agglutination tubes, sealed with vaseline, and incubated 30 minutes at 37°C. The tubes were then immersed for exactly 5 minutes in a constantly agitated water bath at the temperatures shown in the tables. After heating, each tube was cooled immediately in ice water and held under seal until all the heating tests were completed.

1 cc. portions of extract which had been heated at the different temperatures were then aerated by shaking, and further exposed to air in a thin layer at room temperature in the dark. Peroxide tests were made on the heated extract after 1 hour's exposure to air.

0.5 cc. of cell extract which had been heated at these temperatures was added to 1 cc. of a 1:2,000 methylene blue solution in M/30 PO_4. The solutions were mixed thoroughly in small, narrow tubes and then carefully sealed with vaseline. The reduction tests were incubated in a water bath at 37°C. The results are given in Table I.

It is evident (Table I) that heating pneumococcus extract for 5 minutes at 55°C. does not seriously impair either its ability to form peroxide or to reduce methylene blue. On the other hand, heating the same extract for 5 minutes at 65°C. results in practically complete loss of both of these properties, although in the case of the

TABLE I.

Comparison of Effect of Heating for 5 Minutes at Different Temperatures upon the Peroxide-Forming and Methylene Blue-Reducing Activity of Sterile Extracts of Pneumococcus.

Extract.	Peroxide formation after 1 hr. aeration.	Methylene blue reduction after 2 hrs. incubation.
Unheated control	++++*	++++†
5 min. at 55°C.	+++	+++
5 " " 65° "	−	−
5 " " 75° "	−	−
5 " " 85° "	−	−
5 " " 100° "	−	−
Broth (unheated) control	−	−

* ++++ indicates strong peroxide reaction; +++, marked peroxide reaction.

† ++++ indicates complete reduction; +++, almost complete reduction.

methylene blue reaction, delayed and slight reduction was evident after 72 hours.

Extracts heated for 5 minutes at or above 75°C. failed to show any evidence of reducing action even after 3 days. These results indicate that the methylene blue-reducing and peroxide-forming powers of pneumococcus extracts are both susceptible to the destructive action of heat and that each of these activities shows the same rapid increase in rate of destruction when an extract is exposed to temperatures between 55° and 65°C.

2. Effect of Heating at 55°C. upon the Methylene Blue-Reducing Power of Pneumococcus Extracts.—In the following experiment the

rate of destruction of the reducing power is followed by comparisons of the final amount of methylene blue reduced by an extract which had been previously heated for periods of from 5 to 60 minutes at 55°C.

0.5 cc. of the sterile cell extract was placed in a number of narrow tubes, sealed with vaseline, and incubated at 37°C. for 40 minutes. These tubes were then heated anaerobically for different periods at 55°C. in a constantly agitated water bath. Each tube was cooled at once after the heating period and kept under seal until all tubes had been heated.

The concentrations of methylene blue used were 1:3,000, 1:7,500, and 1:15,000. These solutions were brought to constant oxygen tension by saturation with air at

TABLE II.

Influence of Heating Sterile Pneumococcus Extract upon the Total Amount of Methylene Blue Reduced at Final Equilibrium.

Cell extract heated at 55°C. (0.1 cc.).	Reduction of methylene blue (1.4 cc.).		
	1:3,000	1:7,500	1:15,000
Unheated.....	+++*	++++	++++
Heated 5 min.....	++	++++	++++
" 10 "	−	++	++++
" 30 "	−	−	++
" 60 "	−	−	+
Broth control.....	−	−	−
PO_4 solution, control....	−	−	−

* ++++ indicates complete reduction; ++, partial reduction; +, slight reduction; and −, indistinguishable from control.

25°C. 0.1 cc. of heated extract was then added to 1.4 cc. of each concentration of methylene blue solution. As controls, 0.1 cc. of broth, or 0.1 cc. of phosphate solution, was added to each dilution of the dye. All of the reduction tests were sealed with vaseline, and incubated at 37°C.

After 72 hours incubation, the reduction of the dye was determined by comparison with the respective controls.

The data in Table II show that the longer a pneumococcus extract is exposed to 55°C. the less is the amount of dye reduced by equivalent amounts of extract. The gradual decrease in the dye-reducing power of the heated extract may be considered as due to a gradual destruction of the total oxidizing-reducing activity of the

extract. In an analogous experiment in the preceding paper (2), heating an extract at 55°C. was found to effect a gradual decrease in its power to form peroxide. It is evident, therefore, that heating pneumococcus extract for 1 hour at 55°C. results in a gradual loss, but not total destruction, of both its peroxide-forming and methylene blue-reducing properties.

Activation of Sterile Extracts of Washed Pneumococci.

In the preceding paper (2) it was pointed out that thorough washing of pneumococci before extraction deprived the extracts of their capacity to form peroxide on exposure to oxygen. It was further shown that these inactive extracts acquire this property on the addition of the cell washings, muscle infusion, or yeast extract. In the following experiments methylene blue was added to washed cell extracts to determine whether they still retained their reducing action; or if this activity were lost, whether it could be restored again by adding substances known to reactivate the peroxide-forming function of these same extracts.

0.6 cc. of unfiltered, sterile extract of washed pneumococci was added to an equal volume of either muscle infusion or yeast extract which had been previously adjusted to pH 7.5. The test solutions were all brought to uniform oxygen tension by saturation with air at 25°C. To this mixture 0.1 cc. of 1:2,000 solution of methylene blue was added. The small tubes containing the test system were sealed with vaseline and incubated at 37°C. As controls double quantities of washed cell extract alone, and 0.6 cc. quantities of yeast extract or muscle infusion without pneumococcus extract, were added to 0.1 cc. amounts of methylene blue. The total volume was kept constant by the addition of sterile phosphate whenever necessary. The results of this experiment are shown in Table III.

Table III presents evidence that sterile extracts of washed pneumococci are unable by themselves to bring about the reduction of methylene blue. It is further demonstrated in this experiment that washed cell extracts in which reducing activity has ceased, may be stimulated to carry on reduction processes by the addition of muscle infusion and yeast extract.[2] The presence of these activating sub-

[2] Muscle infusion: The infusion was prepared by the routine procedure followed in the laboratory in the preparation of meat infusion broth. 500 gm. of lean beef were allowed to infuse in 1 liter of distilled water overnight at ice box temperature.

stances, which do not themselves reduce the dye, restores to washed cell extracts the power to decolorize methylene blue. This complementing action is wholly analogous to the activation by these same substances of the peroxide-forming power of extracts of washed pneumococci. Harden and Zilva (3) have found that saline suspensions of washed living colon bacilli are by themselves unable to reduce methylene blue, but exhibit this property upon the addition of various substances, among others cell washings, broth, glucose, and peptone.

Influence of Exposure to Air.—When a broth extract of unwashed pneumococci is exposed to air, it suffers a rapid loss of its ability to decolorize methylene blue; if the exposure to oxygen is continued

TABLE III.

Activation of Reducing Activity in Sterile Extracts of Washed Pneumococci.

Sterile extract of washed pneumococci.	Activating substance.		Sterile phosphate solution pH 7.5.	Methylene blue 1:2,000.	Result.	
	Muscle infusion.	Yeast extract.			Reduction.	Time.
cc.	*cc.*	*cc.*	*cc.*	*cc.*		
1.2				0.1	Negative.	5 days.
0.6	0.6			0.1	Complete.	2½ hrs.
	0.6		0.6	0.1	Trace.	5 days.
0.6		0.6		0.1	Complete.	6 hrs.
		0.6	0.6	0.1	Trace.	5 days.

for 12 to 24 hours the methylene blue-reducing action of the extract is completely destroyed. Reference has already been made to the fact that extracts prepared from washed cells are unable to induce reduction of methylene blue in the absence of certain activating substances. Extracts of this nature may be exposed to molecular oxygen for considerable periods with relatively little effect on their

After filtration through coarse paper, the infusion was sterilized for 20 minutes at 100°C. on 3 successive days. The reaction was adjusted to pH 7.5 before use.

Yeast extract: 100 gm. of brewer's yeast in 400 cc. of distilled water were adjusted to pH 4.5 and boiled for 10 minutes. The cellular material was allowed to sediment at room temperature and the clear supernatant was pipetted off and tested for sterility. The extract was stored in the ice box and the reaction adjusted to pH 7.5 before use.

reducing action when subsequently activated by the addition of these complementing substances. If, on the other hand, these same extracts are first activated and then exposed to air, they show a progressive decrease in dye-reducing activity, although the loss is not so pronounced as that which occurs on exposure of the unwashed cell extracts.

The inability of previously oxidized extracts to cause reduction of the dye may be attributable to the loss in activity resulting from the irreversible oxidation of certain constituents of the extract. The loss of this power may also be due in part to the deleterious action on the reducing system, of the peroxide which accumulates in the extract during exposure to air.

Consumption of Molecular Oxygen by Sterile Extracts of Pneumococcus.

From the data in this paper it is evident that sterile extracts of pneumococci cause complete reduction of methylene blue in solutions previously saturated with air. This fact indicates that these extracts are able to consume dissolved oxygen in the presence of methylene blue. However, the production of peroxide by these extracts upon exposure to air is evidence of their ability to consume or accept molecular oxygen in the absence as well as in the presence of methylene blue. Quantitative analyses of oxygen consumption are now being made in a study of this function of the extracts.

DISCUSSION.

In the present work, as in the experiments recorded in the preceding paper, the sterile extracts of pneumococcus employed have been of two types: one type represents broth extracts of unwashed pneumococci; the other, extracts of washed pneumococci which have been extracted in phosphate solution. These two types of extract exhibit marked differences in behavior. The extract prepared from unwashed cells extracted in broth contains a complete oxidizing and reducing system, capable in itself of forming peroxide, or of reducing methylene blue upon the admission of molecular oxygen or the addition of the dye. The second type of extract, prepared from the washed

bacteria cannot by itself initiate either of these reactions without the addition of certain substances found in meat infusion and yeast extract. The presence of these complementary substances "completes" the potentially active system or systems in the washed cell extract and renders it reactive both with molecular oxygen and with methylene blue.

Both types of pneumococcus extract are thermolabile and lose their activity on exposure to 65°C. However, the complementing substances in meat infusion and yeast extract which serve to complete the unheated extracts of washed cells are thermostable. These facts indicate that the active peroxide-forming and methylene blue-reducing systems in pneumococcus extracts consist of two component parts, one sensitive to heat, and the other relatively heat-resistant. Further analysis indicates that the thermolabile component is derived from the bacterial cell and may be ferment-like in nature. The thermostable component can be artificially supplied from sources other than those of bacterial origin. Neither of these components by itself is reactive with molecular oxygen or methylene blue, each being separately inactive and both being essential for the formation of peroxide and the active reduction of the dye. The union of these extract constituents provides an active and complete system in which or by which some substance is oxidized with the formation of peroxide or the reduction of methylene blue, depending upon whether molecular oxygen or the dye functions as hydrogen acceptor. The complex nature of the extracts themselves and especially the unknown character of the substances actually oxidized in the extracts, impose certain limitations on the interpretation and analysis of the phenomena.

In the bacterial extracts employed in these studies, the systems responsible for peroxide production and methylene blue reduction have so many characteristics in common that it seems probable that they are either identical or closely related.

SUMMARY.

1. Sterile broth extracts of unwashed pneumococci, entirely free from living or intact cells, actively reduce methylene blue.

2. Sterile extracts prepared by extracting washed pneumococci in phosphate solution are unable by themselves to reduce methylene

blue. Upon the addition of meat infusion or yeast extract, these washed cell extracts actively reduce methylene blue.

3. The system or systems in pneumococcus extracts responsible for methylene blue reduction are destroyed by exposure to temperatures practically identical with those which have been previously found to destroy the peroxide-forming activity of the same extracts.

4. It is suggested that peroxide formation and methylene blue reduction by pneumococcus extracts are functions of the same or closely related systems, the particular reaction induced depending upon whether molecular oxygen or methylene blue serves as hydrogen acceptor or oxygen donator.

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